Isocitrate-to-SENP1 signaling amplifies insulin secretion and rescues dysfunctional β cells

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Insulin secretion from β cells of the pancreatic islets of Langerhans controls metabolic homeostasis and is impaired in individuals with type 2 diabetes (T2D). Increases in blood glucose trigger insulin release by closing ATP-sensitive K⁺ channels, depolarizing β cells, and opening voltage-dependent Ca²⁺ channels to elicit insulin exocytosis. However, one or more additional pathway(s) amplify the secretory response, likely at the distal exocytotic site. The mitochondrial export of isocitrate and engagement with cytosolic isocitrate dehydrogenase (ICDc) may be one key pathway, but the mechanism linking this to insulin secretion and its role in T2D have not been defined. Here, we show that the ICDc-dependent generation of NADPH and subsequent glutathione (GSH) reduction contribute to the amplification of insulin exocytosis via sentrin/SUMO-specific protease-1 (SENP1). In human T2D and an in vitro model of human islet dysfunction, the glucose-dependent amplification of exocytosis was impaired and could be rescued by introduction of signaling intermediates from this pathway. Moreover, islet-specific *Senp1* deletion in mice caused impaired glucose tolerance by reducing the amplification of insulin exocytosis. Together, our results identify a pathway that links glucose metabolism to the amplification of insulin secretion and demonstrate that restoration of this axis rescues β cell function in T2D.

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Isocitrate-to-SENP1 signaling amplifies insulin secretion and rescues dysfunctional β cells

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Insulin secretion from β cells of the pancreatic islets of Langerhans controls metabolic homeostasis and is impaired in individuals with type 2 diabetes (T2D). Increases in blood glucose trigger insulin release by closing ATP-sensitive K+ channels, depolarizing β cells, and opening voltage-dependent Ca2+ channels to elicit insulin exocytosis. However, one or more additional pathway(s) amplify the secretory response, likely at the distal exocytotic site. The mitochondrial export of isocitrate and engagement with cytosolic isocitrate dehydrogenase (ICDc) may be one key pathway, but the mechanism linking this to insulin secretion and its role in T2D have not been defined. Here, we show that the ICDc-dependent generation of NADPH and subsequent glutathione (GSH) reduction contribute to the amplification of insulin exocytosis via sentrin/SUMO-specific protease-1 (SENP1). In human T2D and an in vitro model of human islet dysfunction, the glucose-dependent amplification of exocytosis was impaired and could be rescued by introduction of signaling intermediates from this pathway. Moreover, islet-specific Senp1 deletion in mice caused impaired glucose tolerance by reducing the amplification of insulin exocytosis. Together, our results identify a pathway that links glucose metabolism to the amplification of insulin secretion and demonstrate that restoration of this axis rescues β cell function in T2D.

Introduction
Insulin secretion from β cells of the pancreatic islets of Langerhans is impaired in type 2 diabetes (T2D) (1). The mechanism by which glucose elicits insulin secretion from β cells involves at least two key pathways (2). A metabolism-dependent closure of ATP-sensitive K+ (KATP) channels (3, 4) elicits action potential firing (5) and activates voltage-dependent Ca2+ channels (VDCCs) (6) to trigger the Ca2+-dependent exocytosis of insulin granules (7, 8). Seminal studies in the 1990s demonstrated an additional “KATP-independent” or “amplifying” action of glucose that is required for optimal secretion (9–11) and controls the amplitude of the secretory response (2). The prevailing evidence suggests that this metabolic amplification of insulin secretion occurs distally in the secretory pathway, possibly at the exocytotic site (12–14).

Multiple metabolic pathways have emerged as potential amplifiers of insulin secretion (13). These include a glycerolipid/free fatty acid cycle (15), which generates monoacylglycerol as a coupling factor (16); a phosphoenolpyruvate and mitochondrial GTP-dependent pathway (17, 18); and so-called “pyruvate cycles” (19–21), which export reducing equivalents from the mitochondria to the cytosol. The mitochondrial export of citrate and isocitrate and subsequent engagement of isocitrate with the cytosolic NADP+-dependent isocitrate dehydrogenase (IDH1), hereafter referred to as ICDc, has been shown to regulate insulin secretion (22, 23), but the metabolites generated by this pathway that engage with the
The glucose-dependent amplification of exocytosis in human β cells is mimicked by metabolic signaling intermediates. Exocytosis in single human β cells, identified by insulin immunostaining, was amplified by glucose stimulation (Figure 1A). In this protocol, we preincubated β cells for 1 hour at low (1 mM) glucose and then stimulated them with 10 mM glucose for approximately 15 minutes, before whole-cell patch clamping to measure the exocytotic response triggered by direct membrane depolarization. This allowed us to measure the ability of prior glucose stimulation to amplify the subsequent exocytic response. Compared with cells kept at 1 mM glucose, 10 mM glucose amplified the exocytic response of β cells to membrane depolarization (Figure 1B) from an average of 8.3 ± 0.6 fF/pF to 26.3 ± 1.3 fF/pF (n = 280 and 311 cells from 50 donors, P < 0.001). This occurred without increasing Ca²⁺ current (Figure 1, C and D, and ref. 38) and is consistent with the view that glucose amplifies insulin exocytosis independent of plasma membrane potential (which was clamped) and Ca²⁺ influx (which was unchanged). The concentration dependence of this amplifying action of glucose was significantly (P < 0.05) left shifted (EC₅₀ = 4.9 ± 0.4 mM, n = 6 donors) compared with its action on insulin secretion (EC₅₀ = 7.2 ± 0.8 mM, n = 6 donors) of isolated human islets (Figure 1E), consistent with an amplifying pathway that operates at glucose levels below the 5 to 7 mM threshold for triggering insulin secretion (11).

We examined whether a pathway downstream of mitochondrial isocitrate export could replicate the amplifying action of glucose. The intracellular dialysis (Figure 2A) of 100 μM isocitrate (Figure 2B) amplified the exocytic response of human β cells to the same extent as 10 mM glucose. The product of the ICDc reaction, α-ketoglutarate (α-KG; 100 μM), did not amplify exocytosis (Figure 2C), and a direct comparison in β cells from the same human donors showed clearly that isocitrate amplified the pathway required isocitrate signaling through ICDc and the generation of NADPH and GSH as coupling factors that act through SENP1 to amplify insulin exocytosis. A role for islet SENP1 as a regulator of in vivo glucose homeostasis was demonstrated by the tissue-selective and inducible knockout of this enzyme. Finally, the glucose-dependent amplification of exocytosis was lost in human T2D β cells but could be rescued by several key intermediates from the proposed isocitrate-to-SENP1 pathway, thereby suggesting new targets for reversing islet dysfunction in T2D.

**Results**

The glucose-dependent amplification of exocytosis in human β cells is mimicked by metabolic signaling intermediates. Exocytosis in single human β cells, identified by insulin immunostaining, was amplified by glucose stimulation (Figure 1A). In this protocol, we preincubated β cells for 1 hour at low (1 mM) glucose and then stimulated them with 10 mM glucose for approximately 15 minutes, before whole-cell patch clamping to measure the exocytotic response triggered by direct membrane depolarization. This allowed us to measure the ability of prior glucose stimulation to amplify the subsequent exocytic response. Compared with cells kept at 1 mM glucose, 10 mM glucose amplified the exocytic response of β cells to membrane depolarization (Figure 1B) from an average of 8.3 ± 0.6 fF/pF to 26.3 ± 1.3 fF/pF (n = 280 and 311 cells from 50 donors, P < 0.001). This occurred without increasing Ca²⁺ current (Figure 1, C and D, and ref. 38) and is consistent with the view that glucose amplifies insulin exocytosis independent of plasma membrane potential (which was clamped) and Ca²⁺ influx (which was unchanged). The concentration dependence of this amplifying action of glucose was significantly (P < 0.05) left shifted (EC₅₀ = 4.9 ± 0.4 mM, n = 6 donors) compared with its action on insulin secretion (EC₅₀ = 7.2 ± 0.8 mM, n = 6 donors) of isolated human islets (Figure 1E), consistent with an amplifying pathway that operates at glucose levels below the 5 to 7 mM threshold for triggering insulin secretion (11).

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**Figure 1. Glucose-dependent amplification of exocytosis in human β cells.**

(A) Exocytotic responses of single human β cells measured as increases in cell membrane capacitance by whole-cell patch clamp (at arrow) performed after acute pretreatment with 1 mM (gray trace) or 10 mM (black trace) glucose (representative of 280 and 311 cells from 50 donors). (B) Cumulative frequency distribution of the exocytotic response in β cells from 50 nondiabetic donors at 1 mM (n = 280 cells) and 10 mM glucose (n = 311 cells). (C) Representative voltage-activated Ca²⁺ currents from human β cells recorded at 1 mM and 10 mM glucose (representative of 72 and 79 cells from 14 donors). (D) The Ca²⁺ charge entry upon a single 500-ms depolarization to 0 mV at 1 mM (n = 72 cells; 14 donors) and 10 mM glucose (n = 79 cells; 14 donors). (E) The glucose concentration-response curve for amplification of the exocytotic response (black) of human β cells (n = 6 donors) is left-shifted compared with that for glucose-stimulated insulin secretion (gray) from intact human islets (n = 6 donors). Data are mean ± SEM and were compared by 2-tailed Student’s t test.
The NADPH-dependent facilitation of insulin exocytosis may occur through its interaction with the glutathione/glutaredoxin system (25). In this model, cytosolic NADPH (but not NADH; refs. 39, 40) acts as a cofactor in the glutathione reductase reaction to maintain a pool of GSH, which, in turn, maintains GRX1 in a reduced state, allowing it to serve as a direct effector of granule exocytosis (24). Using a GSH biosensor (Grx1-roGFP) targeted to the cytosol, we found in human islets that glucose stimulation causes an increase in cytosolic GSH (i.e., a lowering of the oxidation state of this probe; Figure 3D). Since detection of glucose-dependent changes in cytosolic GSH within islets has been difficult using this method (41), we also used HPLC to resolve the reduced and oxidized forms of whole-cell glutathione (GSH and GSSG, respectively) in INS 832/13 cells (Figure 3, E–J). Such measurements are consistent with a requirement for ICDc to generate signals that amplify exocytosis and suggest that NADPH is the key coupling factor in this pathway.

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Figure 3. ICDc is required for glucose-dependent glutathione reduction and the amplification of β cell exocytosis. Knockdown of ICDc (siICDc) in human β cells blunts amplification of exocytosis by (A) glucose (10 mM; n = 24, 41, 18, 49 cells; 7 donors) or (B) isocitrate (100 μM; n = 16, 14, 10, 27 cells; 4 donors), (C) which was rescued by NADPH (10:1 with NADP⁺; n = 16, 33, 29, 26, 31 cells; 5 donors). (D) Oxidation state of Grx1-roGFP expressed in reaggregated human islets (n = 7 donors). (E and H) Normalized to baseline GSSG, GSH is increased by glucose in INS 832/13 cells. (F and I) As GSSG is unchanged, (G and J) the ratio of reduced-to-oxidized glutathione (GSH:GSSG) is increased by glucose. Compared with siScrambled or BSA-treated controls, these responses are lost in INS 832/13 cells following (E–G) knockdown of ICDc (siICDc; n = 9 replicates in 3 experiments) or (H–J) 48-hour culture with 400 μM oleate/palmi-tate (O/P; n = 6 replicates in 2 experiments). (K) Measurement of amino acids in INS 832/13 cells reveals glucose-dependent increases in alanine (Ala) and glutamic acid/glutamine (Glx) and a drop in asparagine/aspartic acid (Asx), which are blocked by aminooxyacetic acid (AOA; n = 3 separate experiments). Intracellular dialysis of GSH amplified exocytosis in (L) INS 832/13 cells (n = 18, 12, 13, 12 cells) and (M) human β cells (n = 33, 24, 14, 49, 15 cells; 5 donors). Data are mean ± SEM and were compared with (A–C, E–K, and M) ANOVA followed by Bonferroni post-test, (D) Wilcoxon matched pairs test, or (L) with the nonparametric Kruskal-Wallis 1-way ANOVA followed by Dunn's post-test. n values correspond to graph bars from left to right, respectively. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control 1 mM glucose condition, unless indicated otherwise.
available for de novo synthesis of glutathione via condensation with cysteine (glutamate-cysteine ligase reaction) and then glycine (glutathione synthetase reaction; Supplemental Figure 2D) and may explain why glutamate also amplifies the β cell exocytotic response (42). Consistent with this, knockdown of ICDc decreased the total pool of glutathione (GSH plus GSSG) in INS 832/13 cells at stimulatory (12 mM) glucose concentrations, from 243.0 ± 22.7 to 165.7 ± 22.4 μmoles/mg protein (P < 0.05, n = 3 experiments).

Previous work demonstrated that GSH can produce a modest enhancement of exocytosis in rat β cells at 5 mM glucose, which is already near the threshold for insulin secretion (25). To test whether GSH is sufficient to amplify the exocytotic response, we examined whether intracellular application of GSH could amplify exocytosis under low glucose (1 mM). The exocytotic response was amplified by intracellular dialysis of GSH (1–100 μM) to the interior of INS 832/13 cells (Figure 3L) and human β cells (Figure 3M). Furthermore, intracellular infusion of 10 μM GSH remained able to amplify exocytosis following knockdown of ICDc (Supplemental Figure 2E).

In addition to serving as a source of NADPH, the pyruvate-isocitrate pathway may contribute to de novo glutathione biosynthesis. In INS 832/13 cells, measurement of amino acids by targeted tandem mass spectrometry revealed that levels of 13 of 16 amino acids were unchanged during glucose stimulation (data not shown). Of the remaining 3 amino acids, the glucose-dependent rise in alanine (Figure 3K) likely resulted from transamination of pyruvate by alanine transaminase. Interestingly, we also observed a glucose-dependent increase in glutamic acid and glutamine and a corresponding decrease in asparagine and aspartic acid (Figure 3K), consistent with a model whereby α-KG produced from the ICDc reaction engages with the cytosolic isoform of aspartate aminotransferase (GOT1) to transaminate α-KG to glutamate (Supplemental Figure 2D). This was blocked by the general transaminase inhibitor aminooxyacetic acid, which also impaired glucose-stimulated insulin secretion in both INS 832/13 cells and primary rat islets (data not shown). The glutamate produced by GOT1 was available for de novo synthesis of glutathione via condensation with cysteine (glutamate-cysteine ligase reaction) and then glycine (glutathione synthetase reaction; Supplemental Figure 2D) and may explain why glutamate also amplifies the β cell exocytotic response (42). Consistent with this, knockdown of ICDc decreased the total pool of glutathione (GSH plus GSSG) in INS 832/13 cells at stimulatory (12 mM) glucose concentrations, from 243.0 ± 22.7 to 165.7 ± 22.4 μmoles/mg protein (P < 0.05, n = 3 experiments).

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SENP1 amplifies exocytosis in β cells downstream of NADPH and GSH. SUMO1 acts as a brake on insulin exocytosis (30), and SENP1’s catalytic activity is reportedly redox dependent (31). Intracellular application of the SENP1 catalytic domain (cSENP1; 4 μg/ml) mimicked the ability of glucose to amplify exocytosis in human β cells (Figure 4A) and rescued the exocytotic response of INS 832/13 cells following knockdown of ICDc (Supplemental Figure 2E).

SENP1 was expressed in human islets, and its mRNA level was unchanged in islets from donors with T2D (Supplemental Figure 3A). SENP1 protein colocalized with insulin granules in both human β cells (thresholded Pearson’s coefficient of 0.61 ± 0.02; n = 34 β cells from 3 donors; Figure 4B) and INS 832/13 cells (thresholded Pearson’s coefficient of 0.68 ± 0.01; n = 47 cells from 4 separate experiments; Figure 4C). Furthermore, when overexpressed in INS 832/13 cells, SENP1-GFP colocalized with secretory granules at the plasma membrane (thresholded Pearson’s coefficient of 0.68 ± 0.04, n = 35 cells in 5 experiments; Figure 4D). No such membrane targeting of SENP1 was observed in HEK293 cells (data not shown).

The catalytic activity of cSENP1 was promoted by GSH and GRX1 (Figure 5A) and treatment of recombinant Flag-SENP1 with the reducing agent DTT exposed free thiols (Figure 5B), which are proposed to be required for SENP1 activity (31). Substitution of cysteines 603 and 613 (with serine) abolishes SENP1 enzymatic activity (43) and oxidation-induced disulfide-mediated dimerization (31) and prevented the SENP1-dependent amplification of exocytosis in INS 832/13 cells (Supplemental Figure 3, B and C).

SENP1 couples redox state to insulin exocytosis. (A) The activity of cSENP1 is enhanced by GSH (10 μM), and this is facilitated by GRX1 (10 μg/ml; n = 3 experiments). (B) Reduction of thiol groups on recombinant Flag-SENP1 expressed in INS 832/13 cells (representative of 3 experiments). (C and D) Stimulation of INS 832/13 cells expressing Flag-SENP1 with 10 mM glucose demonstrates glucose-dependent reduction of SENP1 thiols. (A) A representative blot and (B) quantified data normalized to time = 0 (n = 5 experiments). (E) Native SUMO protease activity in INS 832/13 cells is increased following stimulation of cells with 10 mM glucose (n = 9 experiments). (F) Knockdown of SENP1 in human β cells (insulin, green; SENP1, red; DAPI, blue; representative of 19 and 14 cells) prevents the amplification of exocytosis by NADPH (n = 22, 23, 22, 27, 25, 29 cells; 4 donors) and GSH (n = 26, 28, 27, 36, 34, 44 cells; 5 donors). n values correspond to graph bars from left to right, respectively. Data are mean ± SEM and were compared with ANOVA followed by Bonferroni post-test. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bar: 5 μm (F).

Glucose-dependent amplification of β cell exocytosis is impaired in T2D. Impaired insulin exocytosis is implicated in both in vitro (44) and in vivo (45) rodent models of diabetes, and reduced expression of the exocytotic SNARE proteins has been demonstrated in islets from a limited number of human donors with T2D (46). However, the mechanism underpinning reduced insulin secretion in T2D is unknown, and the connection between impaired mitochondrial function (27, 28, 47) and exocytotic efficiency is unclear. In an in vitro model of human islet dysfunction involving culture of islets with elevated free fatty acids (48), the glucose-dependent amplification of β cell exocytosis was blunted (Figure 6, A and B),
mirroring the impaired insulin secretory response (Figure 6C). In β cells from donors with T2D, glucose was likewise largely unable to amplify exocytosis (Figure 6, D and E). We also confirmed impaired insulin secretion in islets from several donors with T2D (Figure 6F). While glucose stimulation was ineffective, increasing either isocitrate or SENP1 rescued exocytosis in the in vitro model (Figure 6G and H). Similarly, amplification of the exocytotic response in β cells from donors with T2D could be rescued by intracellular infusion of isocitrate (Figure 6I), NADPH (Figure 6J), or GSH (Figure 6K) or by upregulating SENP1 (Figure 6L). These data suggest that the glucose-dependent pathway for amplifying β cell exocytosis is impaired in T2D but can be rescued by several coupling factors generated along the isocitrate-to-SENP1 pathway.

Islet SENP1 regulates glucose homeostasis in vivo by controlling the glucose-dependent amplification of insulin secretion. Reduced expression of SENP1 in islet cells protects against apoptosis (37), while increasing SUMOylation may impair insulin secretion (30, 35). To determine the role of SENP1 in vivo, we generated SENP1fl/fl mice and crossed them with the Pdx1-Cre line (49) to generate a tissue-selective knockout (herein referred to as pSENP1-KO; Figure 7A) mice and with the Pdx-CreER line (50) to generate an inducible β cell–specific knockout (herein referred to as iβSENP1-KO)
mice. SENP1 was severely reduced in islets from either model (Figure 7B and Supplemental Figure 4A), while mRNA of other Senp isoforms was unchanged (Supplemental Figure 4B). SENP1 protein was low in mouse hypothalami (Supplemental Figure 4C), and the body weight of pSENPI-WT and pSENPI-KO animals did not differ (Figure 7C). Compared with littermate controls (Senp1+/+), as was the response to a protocol (9) assessing the KATP-channel mechanism does not define the entire secretory response involving increases in metabolism-derived ATP, inhibition of KATP channels, and activation of VDCCs was first introduced more than 30 years ago (3) and remains as a cornerstone mechanism for the “triggering” of insulin secretion. It is clear, however, that the KATP-channel mechanism does not define the entire secretory response (9–11). Multiple metabolic coupling intermediates have been proposed as factors that amplify the secretory response to a triggering Ca2+ signal (13), with the net export of mitochondrial substrates and reducing equivalents being one strong candidate (19, 20).

Discussion

The canonical mechanism of glucose-stimulated insulin secretion involving increases in metabolism-derived ATP, inhibition of KATP channels, and activation of VDCCs was first introduced more than 30 years ago (3) and remains as a cornerstone mechanism for the “triggering” of insulin secretion. It is clear, however, that the KATP-channel mechanism does not define the entire secretory response (9–11). Multiple metabolic coupling intermediates have been proposed as factors that amplify the secretory response to a triggering Ca2+ signal (13), with the net export of mitochondrial substrates and reducing equivalents being one strong candidate (19, 20). Here, we show that the glucose-dependent amplification of exocytosis in human β cells, which is disrupted in T2D, requires islets to glucosedeleted in pSENPI-KO mice (Figure 7F), despite the fact that islet morphology (Figure 8A) and α cell and β cell mass (Supplemental Figure 5A) were unchanged.

The secretory response of isolated pSENPI-KO islets to glucose and KCl was impaired (Figure 8B and Supplemental Figure 5B), as was the response to a protocol (9) assessing the KATP-independent amplification of insulin secretion (Figure 8C). The insulin content of islets from pSENPI-KO mice was unchanged (Supplemental Figure 5B). A similar phenotype was observed in islets from iSenp1-KO mice (Supplemental Figure 5, C and D) or in response to acute knockdown of SENP1 achieved by treatment of iSenp1-KO mice with an adenovirus expressing the Cre recombinase (Ad-Cre; Supplemental Figure 5, E and F).

In human β cells, knockdown of SENP1 had little effect on action potential dynamics (Supplemental Figure 6, A and B), while action potential firing was only modestly affected in pSENPI-KO β cells (Figure 8D and Supplemental Figure 6C). This is consistent with the SUMO-dependent regulation of Kv2.1 channels (Supplemental Figure 6D and ref. 36), which have a more prominent role in the control of electrical activity in mouse than in human β cells (51). Overall, however, this was not sufficient to alter the [Ca2+]i responses in pSENPI-KO islets (Figure 8E). Rather, impaired insulin secretion results from an impaired glucose-dependent amplification of exocytosis that was rescued by reintroduction of cSENP1 via the patch pipette (Figure 8F). Mouse β cells lacking SENP1 also failed to respond to NADPH (Supplemental Figure 6D) and GSH (Figure 8G). Thus, a reduction of SENP1 in β cells causes glucose intolerance due to an impaired ability of glucose, and its downstream signals, to amplify insulin exocytosis.

Figure 7. Pancreatic islet-selective knockout of Senp1 in mice results in impaired glucose-tolerance and plasma insulin responses. (A) Illustration of the generation of tissue-selective knockout mice. To generate Senp1fl/fl mice, exons 14 and 15 of the Senp1 gene were flanked by 2 loxP sequences. The Senp1fl/fl mice were then crossed with Pdx1-Cre or Pdx1-CreER lines to generate pSENPI-KO and iSenp1-KO mice, respectively. (B) SENP1 protein expression in islets from wild-type (pSENPI-WT) and pSENPI-KO mice (n = 3 mice of each genotype). (C) Body weight of pSENPI-WT (n = 18) and pSENPI-KO (n = 14) mice. (D) Insulin tolerance of the pSENPI-WT (n = 13) and pSENPI-KO (n = 10) littermates was not different. (E) Compared with pSENPI-WT mice (n = 11), pSENPI-KO (n = 9) mice were glucose intolerant following an oral glucose challenge. (F) Plasma insulin responses to oral glucose were blunted in pSENPI-KO mice (n = 6 of each genotype). Data are mean ± SEM and were compared with ANOVA followed by Bonferroni post-test or by 2-tailed Student’s t test (AUC in E and F). *P < 0.05, **P < 0.01, ***P < 0.001.
Glucose affects the magnitude of subsequent secretory responses at concentrations below those required to trigger Ca\(^{2+}\) entry (9, 11). Indeed, the glucose-dependent amplification of exocytosis in human \(\beta\) cells is half maximal at approximately 4.5 mM, less than the corresponding value for inducing insulin secretion from human islets reported here and elsewhere (52, 53). This is consistent with the increased glucose oxidation in human islets at between 1 and 6 mM glucose (54) and suggests that glucose concentrations below the threshold for electrical activity and Ca\(^{2+}\) entry can determine the size of the secretory granule pool available for release. We show that this amplifying action of glucose is lost in \(\beta\) cells in an in vitro model of lipotoxicity and in \(\beta\) cells from Senp1 knockout mice.

Figure 8. Pancreatic islet–specific knockout of Senp1 blunts insulin secretion due to an impaired amplification of exocytosis. (A) Immunostaining of pSenp1-WT and pSenp1-KO mouse pancreatic sections for insulin (green), glucagon (red), and nuclei (blue) revealed no difference in islet architecture (see also Supplemental Figure 3B; \(n = 4\) mice of each genotype). (B) Perfusion profile of the secretory response to glucose (16.7 mM) and KCl (30 mM) of pSenp1-WT and pSenp1-KO islets \((n = 4\) mice of each genotype). (C) With \(K_{\text{ATP}}\) channels held open with diazoxide, the secretory response of pSenp1-WT islets \((n = 4\) mice) to KCl (30 mM) at 16.7 mM (circles) versus 2.8 mM (squares) glucose is blunted in pSenp1-KO islets \((n = 4\) mice). (D) Action potential firing is moderately altered in pSenp1-KO \(\beta\) cells \((n = 17, 19\) cells; 3 and 4 mice of each genotype); see also Supplemental Figure 6, A–C). (E) Although islet intracellular Ca\(^{2+}\) responses were unchanged \((n = 4\) mice of each genotype). (F) The glucose-dependent amplification of exocytosis is lost in pSenp1-KO \(\beta\) cells, and is rescued by reintroduction of cSenp1 (4 \(\mu\)g/ml; \(n = 28, 33, 42, 37, 39\) cells; 4 mice of each genotype). (G) GSH (10 \(\mu\)M) is unable to amplify exocytosis in pSenp1-KO \(\beta\) cells \((n = 37, 39, 57, 51, 32, 49\) cells; 5 mice of each genotype). (H) Proposed pathway linking mitochondrial export of (iso)citrate, glutathione biosynthesis (blue), and glutathione reduction (orange) pathways to the amplification of insulin exocytosis (yellow). Data are mean ± SEM and were compared with ANOVA followed by Bonferroni post-test (B, C, F, and G) or by 2-tailed Student’s t test (D and E). *\(P < 0.05\), **\(P < 0.001\). Scale bar: 100 \(\mu\)m (A). \(n\) values correspond to graph bars from left to right, respectively.
donors with T2D. This likely follows from the impaired glucose utilization (47), oxygen consumption (27, 54), and mitochondrial hyperpolarization (55) reported previously in islets from donors with T2D. Although one outcome of impaired mitochondrial function is reduced ATP generation (55), we found that the impairment of exocytosis in T2D β cells persists when intracellular ATP is high. This is consistent with the view that alternative metabolic coupling factors play a key role in the amplification of insulin secretion (13).

A variety of biochemical and molecular methods has led to evidence supporting a role for pyruvate carboxylase–mediated anaplerotic metabolism of glucose in insulin secretion (13, 19, 20) and the importance of isocitrate engagement with ICDc (22, 23). NADPH produced by this reaction is proposed to increase insulin exocytosis via GRX1 (24, 25). Consistent with this, we found that (a) isocitrate and NADPH were as potent as glucose in amplifying the exocytotic response, while the product of the ICDc reaction, α-KG, was ineffective; (b) siRNA-mediated suppression of ICDc blocked the glucose- and isocitrate-dependent amplification of exocytosis, an effect that could be rescued by NADPH; (c) NADH, an alternate source of reducing equivalents that does not engage with glutathione reductase, was unable to amplify exocytosis; and (d) this pathway connects to glutathione metabolism by maintaining glutathione synthesis and promoting glutathione reduction.

Although one recent study suggests little change in cytosolic GSH while mitochondrial GSH increases upon glucose stimulation of islets (41), we observed herein a reduction of the cytosolic GSH while mitochondrial GSH increases upon glucose stimulation (d) this pathway connects to glutathione metabolism by maintaining glutathione synthesis and promoting glutathione reduction.

Distal mediators of amplification of the insulin secretory response are not well described. Our previous work suggests that SUMOylation acts as a brake on insulin exocytosis, and a glucose-dependent deSUMOylation event at the exocytotic site is required for efficient insulin secretion (30). Intriguingly, the SUMO protease SENP1 (but not the closely related SENP2) is redox dependent (31), consistent with our observations that GSH and GRX1 can increase the activity of this enzyme. The redox sensitivity of SENP1 has been ascribed to key thiols on cysteine 603 (the catalytic residue conserved in all SENPs) and cysteine 613 (unique to SENP1 and SENP5), which we find are required for amplification of exocytosis. A role for the redox-dependent regulation of SENP1 is supported by the opposing actions of H2O2 and GSH/GRX1 on SENP1 activity observed in vitro and our previous finding that oxidation prevents the SENP1-dependent amplification of exocytosis (32).

SUMOylation is suggested to control the activity of the key β cell transcription factors MAFA and PDX1 (57, 58). However, insulin content and islet morphology are unchanged following knock-out of SENP1, and thus, the acute nature by which cSENP1 affects the amplification of exocytosis suggests a direct, rather than transcriptionally mediated, role in insulin secretion. SUMOylation also controls GLP-1 receptor signaling (35), islet survival (37), and glucokinase activity (34). While a role for SENP1 per se (as opposed to other SENPs) in these contexts remains to be investigated, here we have demonstrated a key role for SENP1 in the amplification of insulin exocytosis to control glucose homeostasis in four different models of SENP1 deficiency: by acute knockdown with siRNA in human β cells and by transduction of mouse Senp1β/β cells with Ad-Cre and in both tissue-selective (pSENP1-KO) and tissue-inducible (βSENP1-KO) mouse models. The exact mechanism by which SENP1 augments the distal exocytotic response remains an area of investigation, and several proteins involved in granule trafficking and exocytosis are subject to posttranslational SUMOylation (30, 36, 59–62). We demonstrated previously the glucose-dependent deSUMOylation of synaptotagmin VII (30). DeSUMOylation of synaptotagmin VII is maximal at 15 minutes after glucose stimulation, consistent with the time course of glucose-stimulated SENP1 reduction and SUMO protease activity observed in the present study. Whether deSUMOylation of synaptotagmin VII alone can account for amplification of insulin exocytosis remains unclear; it is likely that SENP1 acts on multiple targets within the exocytotic machinery. Regardless, SENP1 appears to act very near the exocytotic site, given its ability to colocalize with membrane-associated secretory granules and our previous observation that upregulation of SUMO1 blocks exocytosis downstream of insulin granule recruitment.

Thus, we propose (Figure 8H) that glucose stimulation amplifies the secretory response through a pathway that requires the ICDc-dependent generation of NADPH and GSH, which is coupled to enhanced Ca2+-dependent exocytosis by SENP1. Impairment of this signaling pathway in T2D could occur in several ways: (a) reduced mitochondrial function, leading to lowered isocitrate export and cytosolic NADPH production; (b) oxidative stress and redirection of NADPH, GSH, and GRX1 into a protective function; or (c) direct oxidative inactivation of SENP1 (32). That dysfunction in β cells from donors with T2D can be circumvented by reintroduction of isocitrate-to-SENP1 pathway intermediates suggests that the exocytotic mechanism remains intact and could be harnessed for alternative therapeutic approaches to increase insulin secretion in T2D.

Methods
Pancreatic islet isolation and cell culture. Human islets were isolated from donor pancreata at the Alberta Diabetes Institute IsletCore (http://www.bcell.org/IsletCore.html) or the Clinical Islet Laboratory at the University of Alberta as previously described (63) and cultured in low-glucose (5.5 mM) DMEM with l-glutamine, 110 mg/l sodium pyruvate, 10% FBS, and 100 U/ml penicillin/streptomycin. In total, islets from 99 human donors were examined in this study (Supplemental Tables 1 and 2). Diabetes status was determined by patient clinical history and in one case by HbA1c measurement (we took >7.0% HbA1c as indicative of T2D). Mouse islets were isolated by collagenase digestion and cultured in RPMI-1640 containing 11.1 mM glucose with 10% FBS and 100 U/ml penicillin/streptomycin. In all experiments, islets were handpicked for purity. For single-cell experiments, islets were...
dissociated in Ca	extsuperscript{2+}-free buffer, plated in 35-mm dishes, and cultured overnight. INS 832/13 cells were cultured in RPMI-1640 containing 11.1 mM glucose with 10% FBS, 10 mM HEPES, 0.29 mg/ml L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 100 U/ml penicillin/streptomycin, as described previously (64).

Transgenic animals. An ES cell–targeting vector containing exons 14 and 15 flanked by loxp sequence was generated with a neomycin cassette flanked with FRT sequence at the 3′-adjacent region. This was transfected/electroporated into ES cells. The positive ES cells were determined by PCR/Southern blot and then injected into C57BL/6 mice to generate chimeric mice. Mice containing Loxp and Neo were crossed with FLP-FRT mice in order to delete neomycin. Mice containing Loxp without Neo were crossed with Pdx1-Cre mice (49) on a C57BL/6 background [B6.FVB-Tg(Pdx1-cre)6Tuv/J] and Pdx1-CreER mice (50) on a C56BL/6 background [Tg(Pdx1-Cre/Esrt1*)#Dam/J] from The Jackson Laboratory. Experiments were performed on male littermates between 6 and 14 weeks of age as indicated. Ear notches were used to determine mouse genotypes by using the REExtract-Amp Tissue PCR Kit (Sigma-Aldrich) and primers listed in Supplemental Table 3. At 10 weeks, Senp1fl/fl × Pdx-CreER mice and control littermates were injected 3 times with 225 mg/kg tamoxifen prepared in corn oil every 2 days.

Recombinant proteins and plasmid constructs. cSENPI was from Enzo Life Technologies. Recombinant GRX1 and glutathione-S-transferase were from Abcam. Flag-SENP1 was from Addgene (plasmid 17357). Flag-SENP1 mutants were generated by site-directed mutagenesis followed by DpnI digestion using primers listed in Supplemental Table 3. This was confirmed by Sanger sequencing. pEGFP-SENP1 plasmid was generated by cloning the SENP1 cDNA sequence from Flag-SENP1 plasmid into pEGFP backbone (Clontech) using Bgl II and Sal I restriction enzymes (New England Biolabs). Plasmid was transfected in INS 832/13 cells and dissociated human islet cells with Lipofectamine 2000 (Life Technologies).

Recombinant adenosinoviruses. Recombinant adenosinoviruses used to suppress the expression of ICDc (Ad-shICDc) in INS 832/13 cells or deliver a scrambled control shRNA (Ad-shScrambled) were described previously (23, 65). Adenovirus expressing GFP (Ad-GFP), Cre-ires-GFP (Ad-Cre), and GFP-SENP1 (Ad-GFP-SENP1) were from Welgen Inc. Intact and dissociated islets were infected with recombinant viruses as previously described (66).

siRNA-mediated knockdown and quantitative PCR. Sequences and suppliers for siRNA duplexes used are presented in Supplemental Table 3. These were transfected in dissociated human islet cells or INS 832/13 cells using DharmaFECT 1 (GE Healthcare). For quantitative PCR, RNA was extracted using TRIzol Reagent (Life Technologies). cDNA was synthesized using Super Script II and oligo(dT) (Life Technologies) according to the manufacturer’s protocol. Real-time PCR was performed as previously described (37) using primers listed in Supplemental Table 3.

Chemical reagents. Unless stated otherwise, all chemical reagents were from Sigma-Aldrich. Fatty acid–free BSA was from Roche. Fatty acid stock solution was prepared as follows. Oleate and palmitate were dissolved at 70°C in 150 mM NaCl to final concentrations of 13.5 and 6.6 mM, respectively. Fatty acid complexation was achieved by dissolving 1 volume of the previous solution in 1 volume of free fatty acid BSA (8.35% in 150 mM NaCl) at 37°C with stirring for an hour. The stock solution was diluted 10 times in culture media with 1% FBS for a final concentration of 400 μM free fatty acid.

Single-cell measurement of β cell exocytosis, action potentials, and currents. Prior to patch-clamping, dissociated islet cells or INS 832/13 cells were preincubated in DMEM (human) or RPMI-1640 (mouse and INS 832/13) media with 1 mM glucose for 1 hour. Media were then changed to bath solution containing 118 mM NaCl, 20 mM TEA, 5.6 mM KCl, 1.2 mM MgCl	extsubscript{2}-6H	extsubscript{2}O, 2.6 mM CaCl	extsubscript{2}, 5 mM HEPES, and either 1 mM or 10 mM glucose (pH 7.4 with NaOH) in a heated chamber (32°C–35°C). After approximately 15 minutes in the bath solution, whole-cell patch-clamp measurement of exocytosis was performed (30). The intracellular solution contained 125 mM Cs-glutamate, 10 mM CsCl, 10 mM NaCl, 1 mM MgCl	extsubscript{2}-6H	extsubscript{2}O, 0.05 mM EGTA, 5 mM HEPES, 0.1 mM cAMP, and 3 mM MgATP (pH 7.15 with CsOH), and compounds or recombinant enzymes were added to this as indicated for dialysis into the cell. In one set of experiments (Figure 2G), cAMP was omitted and the concentration of MgATP was 0.3 mM. When NADPH or NADH were used, these were 100 μM together with 10 μM of NADP⁺ or NAD⁺, respectively. Exocytosis was stimulated by activating VDCCs with a series of membrane depolarizations from -70 to 0 mV.

Action potentials and voltage–dependent K⁺ currents were measured essentially as described previously (67) using the amphotericin perforated-patch and whole-cell configurations, respectively. Cells were preincubated and then treated with 10 mM glucose as described above for the exocytosis measurements. Following all patch-clamp experiments, cells were positively identified as β cells by insulin immunostaining. Capacitance responses (fF) and K⁺ current (pA) were normalized to initial cell size in picofarad (pf).

Measurement of insulin secretion. Insulin secretion was measured in static secretion assays as described previously (30) or by perfusion at 37°C in Krebs-Ringer buffer (KRB) (115 mM NaCl, 5 mM KCl, 24 mM NaHCO	extsubscript{3}, 2.5 mM CaCl	extsubscript{2}, 1 mM MgCl	extsubscript{2}, 10 mM HEPES, and 0.1% BSA, PH 7.4). For static incubations, 15 handpicked islets were preincubated for 2 hours in 1 mM glucose KRB prior to a 1-hour test period in the same solution, followed by a 1-hour test period in 16.7 mM glucose KRB. For perfusion, 20 islets per lane were perfused (0.1 ml/min) with 2.8 mM glucose KRB for 30 minutes and then with the indicated condition. Islets were lysed in acid/ethanol buffer (1.5% concentrated HCl, 23.5% acetic acid, and 75% ethanol) for total insulin content. Samples were assayed using the Insulin Detection Kit (Meso Scale Discovery).

Glucose and insulin tolerance. Mice were fasted for 4 hours with free access to water. At 6 and 12 weeks, glucose tolerance test was assessed after oral administration of 1 g/kg dextrose by gavage. Blood was collected at the indicated times in Microvette 100 Li Heparin (Sarstedt) and centrifuged at 4°C for 10 minutes at 9,330 × g. At 6 and 12 weeks, glucose tolerance test was assessed after intraperitoneal injection of 1 U/kg Humulin R (Eli Lilly).

Imaging. For immunofluorescence of pancreatic sections, pancreata were fixed with Z-fix (VWR Canada) and embedded in paraffin with sections stained as previously described (68). The total pancreatic section was imaged as a mosaic with a Zeiss Axio Observer.Z1 microscope and ×40 Plan Ap′ × Chromat objective (1.4 NA). Excitation was with a COLIBRI (Carl Zeiss Canada) LED light source with 350-, 495-, or 555-nm filter set. Insulin- and glucagon-positive areas were quantified with ImageJ software (NIH Image). The β and α cell mass were determined as the relative insulin- and glucagon-positive area from 3 to 7 sections of each pancreas normalized to pancreas weight.
Dissociated human islets and INS 832/13 cells cultured on coverslips were fixed with 2×-fix and stained with rabbit polyclonal SENP1 antibody (Acris, AP06456PU, dilution 1:50) overnight at room temperature. Cells were then stained with the guinea pig polyclonal insulin antibody (Dako, A0564, dilution 1:500) for 1 hour at room temperature. Afterward, the Alexa Fluor 594 anti-rabbit (Life Technologies, A-11037) and Alexa Fluor 488 anti-guinea pig (Life Technologies, A-11073) secondary antibodies were applied for an hour. Then, coverslips were mounted with the Prolong Gold Antifade Mountant with DAPI (Life Technologies), and images were acquired using an Olympus AIVI Spinning Disc confocal microscope with a 100× objective. Colocalization was measured using Velocity 3D Image Analysis Software (PerkinElmer).

For total internal reflection fluorescence (TIRF) imaging, INS 832/13 cells were transfected with pEGFP-SENP1 and pmCherry-IAPP plasmids using Lipofectamine 2000 (Life Technologies) and fixed with Z-fix after 48 hours. Imaging was performed with a Cell-TIRF motorized TIRF system (Olympus Canada) equipped with a 100×/1.49 NA TIRFM objective, a Photometrics Evolve 512 camera (Photometrics), and Metamorph Imaging software (Molecular Devices). Stimulation wavelengths were 491 nm and 561 nm, and the penetration depth was set to 105 nm. Colocalization was analyzed with ImageJ (NIH Image) using the JaCoP colocalization plugin.

Calcium imaging of intact mouse islets was performed as previously described (30) using Fura2-AM (Life Technologies).

Fluorescent biosensor measurement of glutathione redox state. A fusion protein (G6X-roGFP) of cytosolic GRX1 and redox-sensitive GFP (roGFP) (69) was transduced into dispersed human islet cells using an adenoviral vector at an MOI of 200 particles per cell. The cells were then reaggregated (5,000 cells per reaggregated islet) by centrifugation at 1,643 g in a 96-well V-bottom plate and cultured for 3 days before being transferred to glass-bottom confocal dishes in 1 mM glucose Krebs buffer. Islets were imaged after a 30-minute preincubation in this buffer and then the glucose concentration was increased to 10 mM before imaging 20 minutes after this addition. For normalization, the fully reduced state was achieved by addition of 5 mM DTT for 15 minutes; the fully oxidized state was achieved by addition of 0.5 mM acivicin to inhibit -glutamyltranspeptidase, and 5 mM N-ethylmaleimide. The 500-μl cell suspension was snap frozen for lysis. The cell homogenate was thawed on wet ice and split into 2 derivatization protocols for the measurement of GSH and GSSG using HPLC (70, 71).

Stability of glucose levels was analyzed with the Prolong Gold Antifade Mountant with DAPI (Life Technologies) and then centrifuged for 5 minutes at 20,000 g. The resulting supernatant (200 μl) was next diluted in 1 ml of 0.1 M NaOH twice to pH 12 before reacting with 0.1% o-phenaldaldehyde to form a fluorescent product detectable at excitation/emission wavelengths of 350/420 nm (Shimadzu RF-20A xs) (70). Derivatized GSSG samples were processed using a 25 mM sodium phosphate buffer containing 15% HPLC-grade methanol at pH 6 and run through a Shimadzu Prominance HPLC system equipped with the same column described above at flow rate of 0.5 ml/min. All samples were quantified using standards prepared under identical conditions and normalized to the protein content measured in the INS 832/13 homogenate by BCA assay.

Measurement of amino acid levels by targeted tandem mass spectrometry. INS 832/13 insulinoma cells, cultured in 6-well plates, were preincubated for 1.5 hours in secretion buffer containing 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.16 mM MgSO4, 20 mM HEPES, and 2.5 mM CaCl2, with 0.2% BSA (pH 7.2), containing 2.5 mM glucose, followed by incubation in secretion buffer for an additional 2 hours with either 2.5 or 12 mM glucose. When indicated, the general transaminase inhibitor aminoxyacetic acid was present at 10 mM during both the preincubation and incubation. Cells were washed 3 times with cold PBS, quick frozen on the plate, and sonicated in H2O on ice. Amino acid levels were measured in the cleared lysates by tandem mass spectrometry as described previously (72, 73).

SUMO protease activity. INS 832/13 cells were preincubated for 3 hours in culture media with 1 mM glucose and for an additional hour in KRB with 1 mM glucose. Cells were then incubated for the indicated time with 10 mM glucose KRB and then lysed in 1x passive lysis buffer (Promega). A fraction of cell extract was added to 500 nM SUMO1-AMC substrate (Enzo Life Science), and fluorescence was monitored over 5 minutes (380 nm excitation, 515 nm emission) using the Wallac Evasion plate reader (Perkin Elmer). Protein concentration was determined by BCA protein assay (Pierce).

For in vitro SENP1 activity, human GRX1 (10 μg/ml, Abcam) and GSH (10 μM, Sigma-Aldrich) were incubated with cSENP1 (50 μM, Boston Biochem) in a buffer containing 20 mM Tris-HCl, 20 mM NaCl, pH 7.8. AMC release reaction was initiated by adding 1 μM SUMO1-AMC substrate (Enzo Life Science), and fluorescence was monitored by fluorimetry, with 380 nm excitation and 460 nm emission wavelengths. The fluorescence reading was recorded with the Synergy 2 Multi-Mode Plate Reader from BioTek for 30 minutes with 1-minute intervals at room temperature.

Western blotting and pull-down experiments. For standard Western blotting, rabbit polyclonal SENP1 antibody (1:2,000) was provided by Mary Dasso (NIH, Bethesda, Maryland, USA) (74). Mouse β-actin antibody (1:1,000) was from Santa Cruz Biotechnology Inc. (sc-47778). Cells were harvested and then lysed in RIPA lysis buffer. Whole-cell lysates were separated using SDS-PAGE, transferred to PVDF membrane, and probed with indicated antibodies.

The thiol groups (or sulhydryl groups) on Flag-SENP1 expressed in INS 832/13 cells were labeled with biotin using 2.5 μM EZ-Link BMCC-biotin (Thermo Scientific) for 1 hour at 4°C. The biotinylated...
Flag-SENP1 was captured with streptavidin-agarose (Thermo Scientific) overnight at 4°C; eluted using 6 M guanidine HCl and 0.1 M glycine, pH 2.5; and subsequently precipitated by methanol chloroform precipitation. The pellet was dissolved in SDS-PAGE loading buffer, and the biotinylated SENP1 was quantified by Western blot using a mouse monoclonal SENP1 antibody (1:500) from Santa Cruz Biotechnology Inc. (sc-271360).

Statistics. Data analysis was performed using FitMaster (HEKA Electronik) and GraphPad Prism (v6.0c). All data are shown as the mean, and error bars represent SEM. For exocytosis data only, statistical outliers were identified by an unbiased ROUT (robust regression followed by outlier identification) test (75) and removed from subsequent analyses. Comparison of multiple groups was performed using ANOVA followed by Bonferroni post-test or the nonparametric Kruskal-Wallis 1-way ANOVA followed by Dunn’s post-test. When comparing two means only, data were analyzed by the 2-tailed Student’s t-test or nonparametric Wilcoxon analysis. A P value less than 0.05 was considered significant.

Study approval. All animal and human islet studies were approved by the Animal Care and Use Committee (AUP00000291, AUP00000405) and the Human Research Ethics Board (Pro00001754) at the University of Alberta. All families of organ donors provided informed consent for use of pancreatic tissue in research.

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